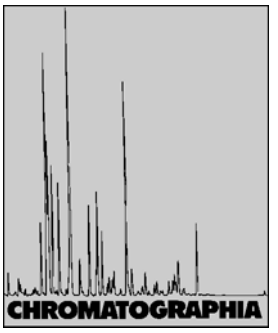
Combined Application of TLC and Matrix-Assisted Laser Desorption and Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) to Phospholipid Analysis of Brain



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Key Words

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Summary

Although matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) was so far scarcely used in phospholipid (PL) analysis, this technique has a great potential: It is fast and reliable, spectra can be quantified and fragmentation of analytes is negligible.

However, individual PL are detected in mixtures with different sensitivities: PL with quaternary ammonia groups are most sensitively detectable while further PL may be suppressed. Therefore, an initial separation of the PL mixture into the individual PL classes is required to be able to detect all PL.

It is the aim of this paper to show on the hand of organic extracts of pig brain as a typical lipid mixture that MALDI-TOF MS in combination with TLC enables the detection of all relevant brain PL. However, it will also be shown that there are problems with the analysis of alkenyl-acyl compounds (plasmalogens) since they decompose in the presence of traces of acids as well as the acidic groups on the TLC plate under formation of the corresponding lysophospholipids.

Introduction

MALDI-TOF MS stands for matrix-assisted laser desorption and ionization time-of-flight mass spectrometry and is – besides electrospray (ESI) – one of the most successful ionisation techniques of modern MS [1]. Both techniques are so-called "soft-ionisation" methods, i.e. they yield only small amounts of fragmentation products, enabling the determination of the molecular mass of the intact analyte molecules [2]. This is a considerable advantage in the field of mixture analysis.

MALDI-TOF MS is commonly used in protein research, in carbohydrate and DNA analysis [1]. However, there is to date only a very limited number of applications in phospholipid (PL) analysis [3–6]. This is surprising if one considers the high reproducibility of spectra that enables even quantitative analysis [7].

However, one serious drawback of MALDI-TOF MS is that not all PL classes are detected with the same sensitivity [8]: PL with quaternary ammonia groups like phosphatidylcholine (PC), sphingomyelin (SM) and derivatives like lyso-PL are most sensitively detected and may suppress other PL [8]. Because of the low detectability of PC and related PL as negative ions [9], this is not a major problem for acidic PL like phosphatidylserine (PS) and phosphatidylinositol (PI) that give also intense signals as negative ions. However, phosphatidylethanolamine (PE) causes considerable problems since PE gives only a weak signal as negative ion but is completely suppressed as positive ion in the presence of PC [8]. Therefore, a previous separation is necessary. Because of its rapid performance, thin-layer chromatography (TLC) is often used for the separation of PL [10]. However, combinations between MALDI-TOF MS and TLC in PL research were so far only scarcely described [3].

In this study an organic extract of pig brain is used as a typical example of a complex PL mixture. The PL composition of brain is of considerable interest since, for instance, Alzheimer's disease is characterised by PL abnormalities [11]. It will be shown that the combination between TLC and MALDI-TOF MS is useful in order to detect all individual PL classes even in complex mixtures.

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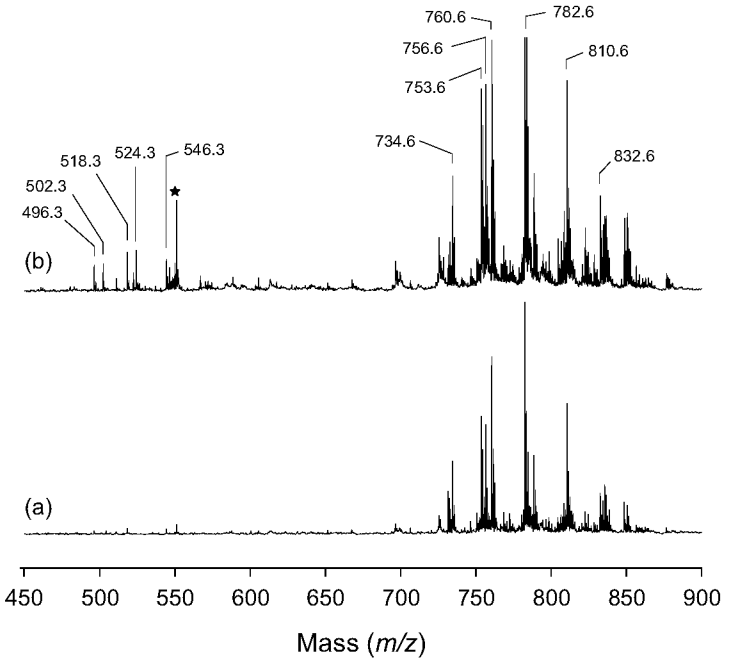

Figure 1. Positive ion MALDI-TOF mass spectra of organic extracts of pig brain. Spectrum (**a**) was recorded in the absence of TFA and (**b**) in the presence of 0.1% TFA. Peaks are labelled according to their *m/z* ratios. The peak marked with an asterisk is caused by the matrix and is formed upon oligomerisation of the applied DHB molecule. For further details see text.

However, it will also be shown that the separation of plasmalogens (alkenyl-acyl compounds of PE and to a lesser extent of PC) that are major constituents of brain PL [12] must be very carefully performed since plasmalogens are decomposed on the TLC plate upon separation – most probably due to the acidic functional groups of the silica gel [13]. Finally, it will be shown that trifluoroacetic acid that is routinely used in MALDI-TOF MS to increase the signal-to-noise ratio [1, 8] must not be used when plasmalogens are of interest.

Experimental

Chemicals

All chemicals for brain extraction and buffer preparation, MALDI-TOF MS (2,5-dihydroxybenzoic acid (DHB) and trifluoroacetic acid (TFA)) and all solvents (chloroform, acetone and methanol) were obtained in highest commercially available purity from Fluka Feinchemikalien GmbH (Taufkirchen, Germany).

Phospholipase A_2 (from hog pancreas) showing an activity of 561 U mg^{-1} was purchased as lyophilizate from Fluka, whereas the dye PRIMULINE was from Aldrich.

Phospholipid Extraction from Brain

Brains of juvenile pigs were obtained from a local slaughterhouse. No differentiation between the individual regions (e.g. white and grey matter) was performed and the brains were homogenised prior to extraction in a portland mortar.

The lipid extraction was performed according to the method of Folch [14]. Typically, about 400 mg brain tissue were mixed with 4 mL of a $CHCl_3/CH_3OH$ mixture (2:1, *v/v*) and vigorously stirred for 20 min. Afterwards the mixture was filtered through paper filter and 1.2 mL 0.9% NaCl in water was added. After centrifugation, the (lower) $CHCl_3$ phase was used for all further experiments. Lipid extraction was also performed with slight modifications of this procedure, but differences in PL composition were weak (data not shown).

Artificial Modifications of PL Extracted from Brain

In some cases, PL from pig brain were digested by phospholipase A_2 (PLA_2) to obtain the corresponding lysophospholipids (LPL) [15] that allow further conclusions on fatty acid compositions of PL since they contain only one fatty acid residue. Briefly, aliquots of pig brain extracts were evaporated to dryness [15]. Vesicles were prepared by dissolving the resulting PL film in 10 mM phosphate buffer pH 7.4 and vortexing vigorously for 30 s. Vesicles were treated with 0.1 mg mL^{-1} phospholipase A_2 and incubated for 2 h at 37 °C. PL were subsequently extracted as described above.

To make assignment of alkenyl-acyl-PL unambiguous, dried PL samples were exposed 10 min to HCl fumes by keeping the inverted flask over an open bottle of concentrated HCl [16].

MALDI-TOF Mass Spectrometry

A 20 µL aliquot of the organic pig brain extracts was mixed with 20 µL of the corresponding matrix solution. For most samples a 0.5 mol L^{-1} 2,5-dihydroxybenzoic acid (DHB) solution in methanol containing 0.1% trifluoroacetic acid (TFA) was used [4]. In selected cases, TFA was omitted. Measurements were also possible in the absence of TFA but on the cost of a worse signal-to-noise ratio. Subsequently, samples were brought onto the sample plate and dried under a warm air stream.

All MALDI-TOF mass spectra were acquired on a Voyager Biospectrometry DE workstation (PerSeptive Biosystems, Framingham, MA, USA). The system utilises a pulsed nitrogen laser, emitting at 337 nm. The extraction voltage was 20 kV and the "low-mass gate" was turned on to prevent the saturation of the detector by ions resulting from the matrix [8]. 128 single laser shots were averaged for each mass spectrum. The laser strength was kept about ten percent above threshold to obtain optimum signal to noise ratio. In order to enhance the spectral resolution all spectra were acquired in the reflector mode. A more detailed methodological description is given in [17].

Separation of PL by Thin-Layer Chromatography

TLC was performed using HPTLC silica gel 60 plates (10 × 10 cm in size) from Merck (Darmstadt, Germany). The elution solvent was chloroform, ethanol, water, triethylamine (30:35:7:35, *v/v/v/v*). The TLC chamber was provided by CAMAG (Switzerland). 5 µL PL samples were used.

Since characterisation of individual PL was planed by MALDI-TOF MS, no vi-

sualisation (e.g. by dye binding) that would result in a change of the molecular weight could be performed. Therefore, PL were visualised by spraying with a solution of PRIMULINE (Direct Yellow 59) in acetone/water (80:20, *v/v*) according to [18]. Upon irradiation by UV light (254 nm), PL can be made visible as violet spots. These individual spots were carefully scratched off and the PL eluted by the addition of a mixture of 75 µL $CHCl_3$, 75 µL methanol and 75 µL 0.9% NaCl in water and intense vortexing. Afterwards, samples were centrifuged (2500 U min^{-1}) to allow phase separation. The organic layer was evaporated to dryness and the residual material redissolved in 20 µL matrix solution and directly used for MALDI-TOF MS.

Results and Discussion

Brain is a lipid-rich tissue that is characterised by a complex PL composition [12]. The interest in PL analysis of brain is coming from pathologies like Alzheimer's disease that are assumed to correlate with abnormalities of PL composition [12]. Therefore, in this paper we will focus exclusively on PL analysis and other brain lipids like cholesterol, gangliosides and cerebrosides will not be considered.

Selected positive ion MALDI-TOF mass spectra of organic extracts of pig brain are shown in Figure 1. Trace (1a) corresponds to the spectrum recorded in the absence of trifluoroacetic acid (TFA), whereas (1b) was recorded in the presence of 0.1% TFA. This concentration of TFA is used in routine MALDI-TOF MS to enhance the ion yield and, accordingly, the sensitivity [1, 4].

Although both spectra (Figure 1) resemble each other closely, it is obvious that the presence of TFA leads to minor alterations in the peak pattern and is especially accompanied by enhanced concentrations of LPL (cf. left end of spectra and Table I for peak assignment). The LPL generation that occurs under these conditions will be discussed in more detail because by TLC significant amounts of LPL could not be detected. Since we have already shown in previous communications that the laser irradiation used in MALDI-TOF MS does not lead to enhanced LPL generation [19], LPL have to be generated by another process.

The most intense peaks in Figure 1 can be easily assigned to different PC species:

Table I. Overview of the observed *m/z* values in the positive ion MALDI-TOF mass spectra of organic extracts of pig brain and their corresponding assignment to individual compounds. Abbreviations: PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; SM, sphingomyelin; LPE, lyso-phosphatidylethanolamine and LPC, lyso-phosphatidylcholine. One should note that all PL besides SM and PC contain functional groups showing exchange with the solvents and/or ions in the matrix solution.

Peak Position	Assignment of Molecular Mass
464.3	1-Octadecenyl-2-hydroxy-PE ($+H^+$)
466.3	1-Octadecyl-2-hydroxy-PE (H^+)
480.3	LPE 18:1 ($+H^+$)
482.3	LPE 18:0 ($+H^+$)
486.3	1-Octadecenyl-2-hydroxy-PE ($+Na^+$)
488.3	1-Octadecyl-2-hydroxy-PE ($+Na^+$)
496.3	LPC 16:0 ($+H^+$)
502.3	LPE 20:4 ($+H^+$)/LPE 18:1 (+Na+)
504.3	LPE 18:0 ($+Na^+$)
518.3	LPC 16:0 ($+Na^+$)
522.3	LPC 18:1 ($+H^+$)
524.3	LPC 18:0 ($+H^+$) or LPE 20:4 ($+Na^+$)
526.3	LPE 18:0 ($-H^+ + 2Na^+$)
544.3	LPC 18:1 ($+Na^+$)
546.3	LPC 18:0 ($+Na^+$)
551.0	Oligomerisation Product of DHB Matrix
664	Impurity arising from plastic material
686	Impurity arising from plastic material
724.6	PE 16:0 (alkyl) /18:1 ($+Na^+$)
727.0	Oligomerisation Product of DHB Matrix
731.6	SM 18:0 ($+H^+$)
734.6	PC 16:0/16:0 ($+H^+$)
746.6	PE 16:0 (alkyl)/20:4 ($+Na^+$)
748.6	PE 16:0 (alkyl)/22:6 ($+H^+$)
753.6	SM 18:0 ($+Na^+$)
756.6	PC 16:0/16:0 ($+Na^+$)
760.6	PC 16:0/18:1 ($+H^+$)
770.6	PE 16:0 (alkenyl)/22:6 ($+Na^+$)
774.6	PE 18:0 (alkyl)/20:4 ($+Na^+$)
781.6	SM 20:0 ($+Na^+$)
782.6	PC 16:0/18:1 ($+Na^+$)
788.6	PC 18:0/18:1 ($+H^+$)
790.6	PE 18:0/20:4 ($+Na^+$)
810.6	PC 18:0/20:4 ($+H^+$) or PC 18:0/18:1 ($+Na^+$)
812.6	PS 18:0/18:1 ($+H^+ + Na^+$)
813.6	SM 24:1 ($+H^+$)
814.6	PE 18:0/22:6 ($+Na^+$)
832.6	PC 18:0/20:4 ($+Na^+$)
834.6	PC 18:0/22:6 (H^+) or PS 18:0/18:1 ($+2\,Na^+$)
835.6	SM 24:1 ($+Na^+$)
836.6	PE 18:0/22:6 ($-H^+ + 2Na^+$)
856.6	PC 18:0/22:6 ($+Na^+$) or PS 18:0/18:1 ($-H^+ + 3Na^+$)
858.6	PS 18:0/22:6 ($+H^+ + Na^+$)
880.6	PS 18:0/22:6 ($+2Na^+$)
902.6	PS 18:0/22:6 ($-H^+ + 3Na^+$)
903.6	PI 18:1/18:1 ($+2Na^+$)
931.6	PI 18:0/20:4 ($+2Na^+$)

The peaks at *m/z* = 760.6 and 782.6 are caused by the proton and the sodium adduct, respectively, of 1-palmitoyl-2-oleoyl-PC, whereas the peaks at *m/z* = 810.6 and 832.6 are caused by 1-stearoyl-2-arachidonoyl-PC and at *m/z* = 734.6 and 756.6 by dipalmitoyl-PC. Finally, the peak at *m/z* = 753.6 corresponds to the sodium adduct of sphingomyelin 18:0 [8]. The ratio between the proton and sodium adducts is determined by the ion composition of the solvents and can be easily changed [20]. A more comprehensive assignment of all detected peaks is given in Table I.

It is a well-known property of MALDI-TOF MS that in the presence of higher amounts of PL with quaternary ammonia groups, other PL may be suppressed [8]. Therefore, spectra in Figure 1 are dominated by PC and SM and a previous separation is necessary to detect further PL. We have used TLC for separation and MALDI-TOF MS for PL detection. This was simply done by scratching off the spots of interest from the TLC plate and eluting the individual PL with organic solvents (see Materials & Methods for details).

Figure 2 shows the positive ion MALDI-TOF mass spectra of individual

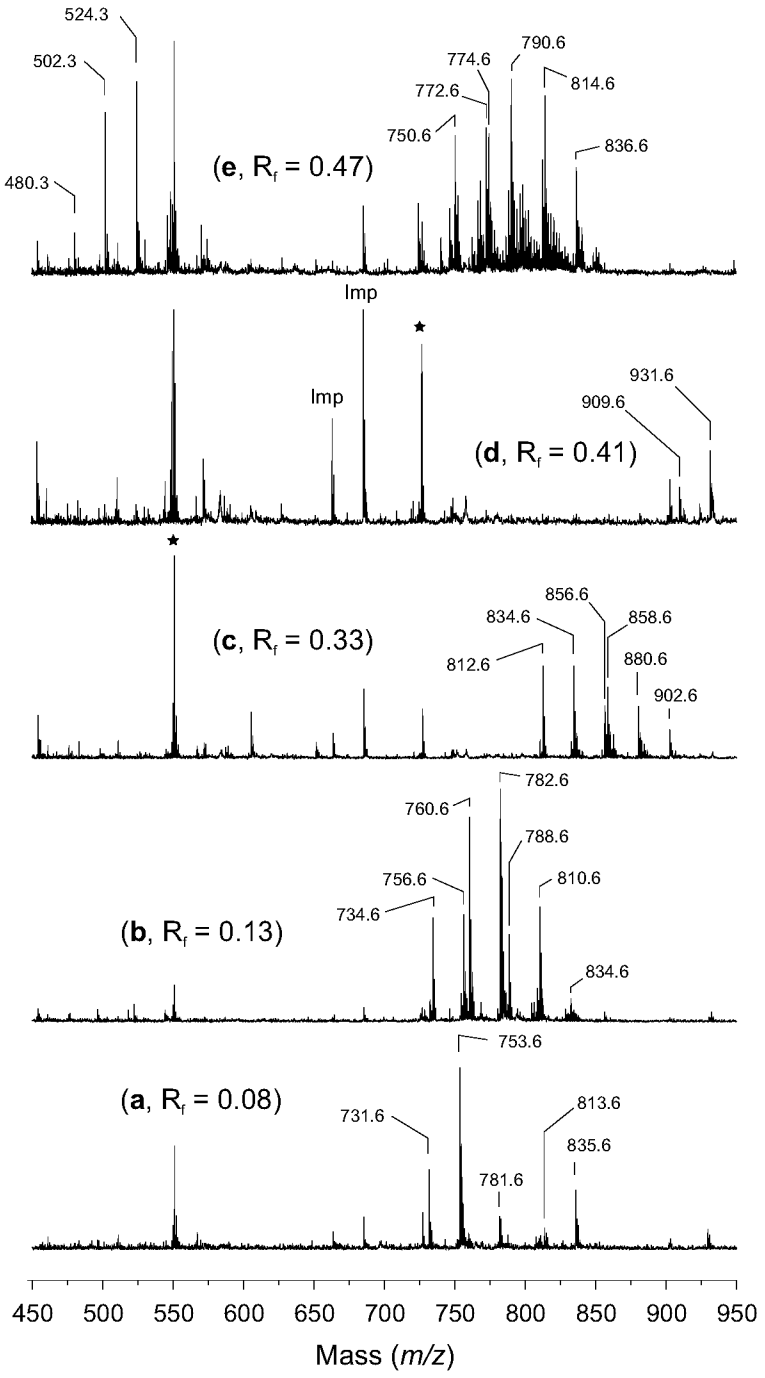

Figure 2. Positive ion MALDI-TOF mass spectra of individual PL fractions obtained by TLC of an organic extract of pig brain. TLC was performed in the absence of acids. Individual spots were scraped off from the TLC plate and eluted with organic solvents. Individual spectra correspond to the SM (**a**), PC (**b**), PS (**c**), PI (**d**) and PE (**e**) fraction. Peaks are labelled according to their *m/z* ratios. The asterisks represents a characteristic matrix peak and the abbreviation "Imp" means an impurity stemming most probably from plastic material present in organic extracts.

PL classes of pig brain recorded after TLC separation as well as the corresponding R_f values. Although there were actually eight fractions, we will focus on the discussion of five selected fractions representing the most relevant PL of brain.

All spectra shown in Figure 2 were recorded in the absence of TFA. Trace (2a) corresponds to the sphingomyelin fraction, trace (2b) to PC, trace (2c) to phosphatidylserine (PS), trace (2d) to phosphatidylinositol (PI) and (2e) to the phosphatidylethanolamine (PE) fraction, respectively. According to their occurrence in brain and their charge characteristics, individual PL are detected with different sensitivities [21]. This is obvious if the intensity of the characteristic matrix peaks at *m/z* = 551 and *m/z* = 727 (marked with asterisks) are compared with the peak of interest [19].

It is obvious that the different PL fractions are characterised by strongly varying fatty acid distributions, with PE (2e) having the most complex composition. In contrast, the fatty acid composition of SM (2a) is most simple: SM contains primarily stearic acid (18:0) and this is clearly reflected by the peaks at *m/z* = 731.6 and 753.6 corresponding to the proton and the sodium adduct, respectively. In smaller amounts there is also SM 24:1 (*m/z* = 813.6 and 835.6) and 20:0 (*m/z* = 781.6). In contrast, the PC fraction (2b) consists primarily of dipalmitoyl-PC (*m/z* = 734.6 and 756.6), palmitoyl-oleoyl-PC (*m/z* = 760.6 and 782.6), stearoyl-oleoyl-PC (*m/z* = 788.6 and 810.6) and small amounts of PC with highly unsaturated fatty acid residues (cf. Table I).

The PS fraction (2c) is primarily composed of highly unsaturated fatty acids: PS 18:0/18:1 ((*m/z* = 812.6, 834.6, and 856.6) and PS 18:0/22:6 (*m/z* = 858.6, 880.6 and 902.6) represent the most abundant compounds. The fact that PS is in contrast to the other PL a negatively-charged PL, is clearly reflected by the more complicated adduct pattern since more than one cation is necessary for charge compensation to obtain a single positively-charged ion. Of course, PS can be also detected in the corresponding negative ion spectra (data not shown).

Because of its rather low occurrence in brain, PI gives rather weak peaks (2d). There are two dominating species, namely the sodium adducts of PI 18:1/18:1 (*m/z* = 909.6) and PI 18:0/20:4 (*m/z* = 931.6). The reader should also note that in trace (2c) and (2d) there are also intense peaks at *m/z* = 664 and 686 (marked with "Imp") that are not caused by PL. These peaks are caused by impurities and represent most probably plasticers that are leached from plastic material and/or the TLC plates [1, 3]. These peaks are nearly always detectable but are by far smaller when more concentrated PL solutions or more easily detectable PL like PC are investigated.

Obviously, the fatty acid distribution of the PE fraction (2e) is the most complex under all PL fractions. PE comprises besides diacyl-PE also significant amounts of alkenyl-acyl compounds, the so-called plasmalogens. The most abundant PE species of brain are listed in Table I. In contrast to all other PL classes, the PE fraction contains also significant amounts

of lyso-phosphatidylethanolamine (LPE) that lacks one fatty acid. This is surprising since LPE should yield an additional TLC fraction because of its different polarity in comparison to PE [10].

Additionally, the fatty acid composition of the LPE is rather unusual and comprises mainly unsaturated fatty acid residues: The most intense peaks at *m/z* = 480.3, 502.3 and 524.3 correspond to LPE 18:1 as well as LPE 20:4 (*m/z* = 502.3 and 524.3). We suppose that LPE is not present as such in the PE fraction but is generated by the decomposition of alkenyl-acyl-PE on the TLC plate. A comparable degradation mechanism of acid-sensitive compounds by the acidic groups on the TLC plate was recently reported [13]. Diacyl-PE and alkenyl-acyl-PE can be separated from each other by means of two-dimensional TLC (data not shown) [10].

In order to clarify the assignment of individual PE species, the total brain extract was digested on the one hand with phospholipase A_2 (PLA_2) and on the other hand treated with HCl fumes [16]. The comparison of both treatments should allow further conclusions on the fatty acid composition of the individual PL since PLA_2 cleaves selectively the fatty acid in *sn*-2 position, whereas HCl fumes hydrolyse the ether bond in alkenyl-acyl-PL, i.e. cleave the fatty acid residue in *sn*-1 position [16].

In Figure 3 the positive ion MALDI-TOF mass spectra of TLC-separated LPL obtained by digestion of the total pig brain extract with PLA_2 (left) or exposition to HCl fumes (right) are shown. Traces (3a) and (3b) represent the spectra of the LPE and (3c) and (3d) the corresponding LPC fraction. We have chosen these both PL classes for a more comprehensive analysis since they represent the most frequently occurring PL of brain [12].

It is obvious that individual spectra provided in Figure 3 differ considerably in dependence on the pre-treatment and – of course – which LPL fraction was used for investigation. There are only very slight differences in the peak patterns of the generated LPC even if they are generated by PLA_2 digestion (3c) or by treatment with HCl fumes (3d). Under both conditions, primarily LPC 16:0 (*m/z* = 496.3 and 518.3) as well as LPC 18:0 (*m/z* = 524.3 and 546.3) are generated. This indicates that primarily the unsaturated fatty acid residue in *sn*-2 position is cleft, resulting in the formation of the corresponding saturated LPC.

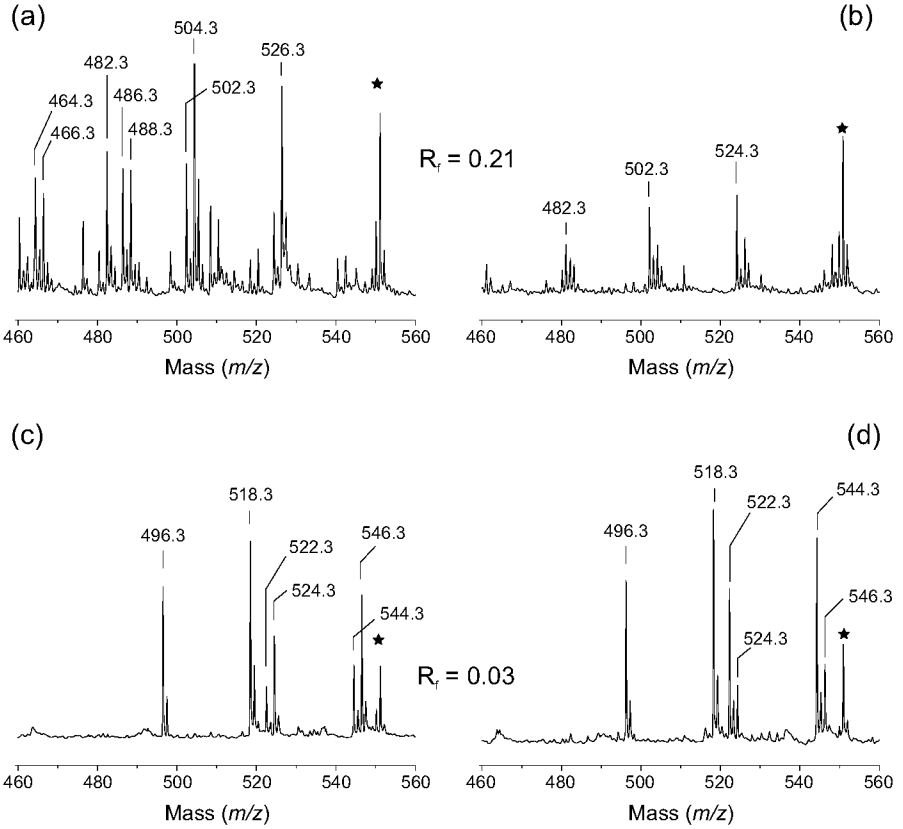

Figure 3. Positive ion MALDI-TOF mass spectra of organic extracts of pig brain pre-treated with PLA_2 or HCl and afterwards subjected to TLC. TLC was performed in the absence of acids. On the left side the total lipid extract was digested with phospholipase A_2 and on the right side with HCl (see Experimental). Traces (**a**) and (**b**) represent the LPE fractions and (**c**) and (**d**) the corresponding LPC fractions. Peaks are labelled according to their m/z ratios and the asterisk represents a characteristic matrix peak.

However, there is obviously more LPC 18:1 (*m/z* = 522.3 and 544.3) in the sample that was treated with HCl (3d) than in the sample digested with PLA_2 (3c). The occurrence of LPC 18:1 might be caused by the hydrolysis of plasmalogens of PC, but compounds with a characteristic mass shift in comparison to standard LPC should be also detectable upon PLA_2 digestion. Since this is not the case (3c) we suggest that not only plasmalogens but also diacyl-PC are (at least partially) affected by HCl fumes. This possibility is currently investigated in our laboratory in dependence on the degree of unsaturation of individual diacyl-PC.

The LPE fractions (3a and 3b) show considerable differences in comparison to the LPC fractions and there are also clear differences when the total brain extract is subjected to PLA_2 digestion (3a) or exposed to HCl fumes (3b): In the first case, a highly complicated mixture is generated, while upon HCl-treatment by far less different LPE species can be detected. The main products upon treatment of the total brain lipid extract with HCl fumes are LPE 20:4 (*m/z* = 502.3 and 524.3) as well as LPE 18:1 (*m/z* = 480.3, 502.3 and 524.3) and these products are stemming from plasmalogen degradation since they contain unsaturated fatty acid residues. The contribution of LPE with saturated fatty acids is by far lower.

In contrast, the LPE obtained upon PLA_2 digestion of the total brain lipid extract (3a) exhibit a more complex peak pattern since under these conditions all compounds equally if alkenyl-acyl or diacyl-PE are cleft in *sn*-2 position.

Therefore, it is our conclusion that combined MALDI-TOF MS-TLC in combination with further enzymatic or chemical treatment is a useful approach to characterise even complex PL mixtures from biological tissues in a relatively short time. However, great caution is needed when plasmalogens are present in the mixture since such compounds decompose on the TLC plate under the generation of the corresponding lysophospholipid.

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